Endotoxin-free plasmid DNA purification

User manual

NucleoBond® 96 Xtra EF

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MACHEREY-NAGEL
www.mn-net.com

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1 Components

1.1 Kit contents

	NucleoBond® 96 Xtra EF	
	1 x 96 preps	**4 x 96 preps**
REF	**740430.1**	**740430.4**
Buffer RES-EF	100 mL	2 x 100 mL
Buffer LYS-EF	100 mL	2 x 100 mL
Buffer NEU-EF	100 mL	2 x 100 mL
Buffer EQU-EF	100 mL	400 mL
Buffer ENDO-EF	200 mL	2 x 400 mL
Buffer WASH-EF	100 mL	500 mL
Buffer ELU-EF	60 mL	300 mL
Buffer TE-EF	125 mL	500 mL
80 % EtOH (Concentrate)	50 mL	200 mL
H_2O-EF	30 mL	125 mL
RNase A* (lyophilized)	3 mg	2 x 6 mg
Culture Plate (with Gas-permeable Foil)	1	4
Square-well Block	2	8
Elution Plate U-bottom (with Self-adhering PE-Foil for sealing)	1	4
NucleoBond® Xtra EF Plate	1	4
NucleoBond® Filter Plate (light orange rings)	1	4
NucleoBond® Finalizer Plate (red rings)	1	4
MN Wash Plate	1	4
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* For preparation of working solutions and storage conditions see section 3.

1.2 Reagents, consumables, and equipment to be supplied by user

Reagents

- 96–100% ethanol
- Isopropanol

Consumables

- Disposable pipette tips

Equipment

- Manual pipettors
- NucleoVac 96 Vacuum Manifold
- 96-well plate or tube centrifuge for harvesting bacterial cells

2 Product description

2.1 The basic principle

The **NucleoBond® 96 Xtra EF** procedure combines the most effective alkaline lysis with gravity-flow anion-exchange chromatography and fast vacuum filtration of isopropanol precipitated plasmid for extremely fast high-throughput purification of endotoxin-free plasmid DNA.

Bacteria are cultivated in the 96-well Culture Plate or glass tubes and harvested by centrifugation. The pelleted cells are resuspended and lysed according to a modified version of the Birnboim and Doly* plasmid Mini prep protocol under alkaline conditions.

The resulting crude lysates are cleared under vacuum with a NucleoBond® Filter Plate and loaded onto the NucleoBond® Xtra EF Plate by gravity flow. Extensive washing of the silica based anion-exchange matrix removes RNA, protein, carbohydrates, and endotoxins completely.

The DNA is eluted in a high-salt buffer that requires a final clean-up by isopropanol precipitation. The precipitated plasmid DNA is loaded by vacuum filtration onto the NucleoBond® Finalizer Plate, washed and finally eluted in endotoxin-free water or TE buffer.

2.2 Kit specifications

Kit specifications at a glance	
Parameter	**NucleoBond® 96 Xtra EF**
Sample material	1–5 mL *E. coli* culture
Vector size	< 15 kbp < 300 kbp (without NucleoBond® Finalizer Plate)
Typical yield	2–4 µg/mL (1.5 mL LB/TB in 96-well culture plates) 10–50 µg/mL (5 mL LB/TB in glass tubes)
A_{260}/A_{280}	1.80–1.95
Elution volume	100–200 µL
Binding capacity	50 µg
Endotoxin level	< 0.1 EU/µg plasmid DNA
Preparation time	120 min/plate

* Birnboim, H.C. & Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523.

2.3 NucleoBond® Xtra purification system

NucleoBond® Xtra Silica Resin is a patented silica-based anion-exchanger, developed by MACHEREY-NAGEL for routine separation of different classes of nucleic acids such as oligonucleotides, RNA, and plasmids.

It consists of hydrophilic, macroporous silica beads functionalized with MAE (methyl-amino-ethanol). The dense coating of this functional group provides a high positive charge density under acidic pH conditions that permits the negatively charged phosphate backbone of plasmid DNA to bind with high specificity (Figure 1).

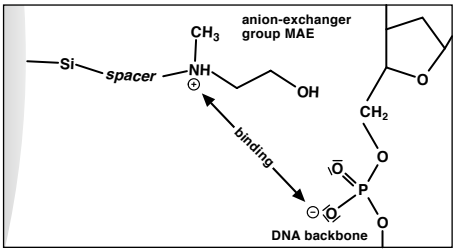


Figure 1: Ionic interaction of the positively charged methyl-hydroxyethyl-amino (MA) group with the negative phosphate oxygen of the DNA backbone.
In contrast to the widely used DEAE (diethylaminoethyl) group, the hydroxy group of MA can be involved in additional hydrogen bonding interactions with the DNA.

Due to a specialized manufacturing process which is strictly controlled and monitored, the **NucleoBond® Xtra** silica beads are uniform in diameter and contain particularly large pores. These special properties allow optimized flow rates and sharp, well-defined elution profiles. **NucleoBond® Xtra** can separate distinct nucleic acid species from each other and from proteins, carbohydrates, and other unwanted cellular components over an exceptionally broad range of salt concentrations (Figure 2).

All contaminants from proteins to RNA and especially endotoxins are washed from the column. The positive charge of the resin is neutralized by a pH shift to slightly alkaline conditions, and pure plasmid DNA is eluted in a high-salt elution buffer.

The purified nucleic acid products are suitable for use in the most demanding molecular biology applications, including extremely sensitive transfections, *in vitro* transcription, automated or manual sequencing, cloning, hybridization, and PCR.

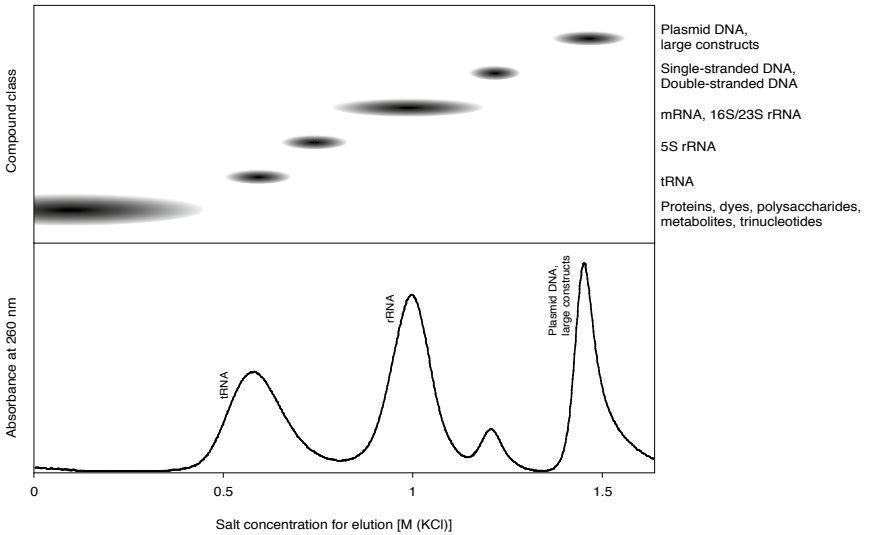


Figure 2: Elution profile of NucleoBond® Xtra Silica Resin at pH 7.0
The more interactions a nucleic acid can form between the phosphate backbone and the positively charged resin, the later it is eluted with increasing salt concentration. Large nucleic acids carry more charges than short ones and double-stranded DNA more than single-stranded RNA.

2.4 Endotoxins

2.4.1 Localization, molecular structure, and function of endotoxins

In contrast to Gram-positive bacteria which have only one lipid bilayer membrane surrounded by a thick cell wall, Gram-negative bacteria have a second membrane enclosing the inner membrane and only a thin cell wall. The outer layer of this second membrane consists of amphiphilic lipopolysaccharides (LPS), also called endotoxins.

The structure of endotoxins can be divided into three domains:

1. The hydrophobic **Lipid A moiety** is anchoring the LPS inside the membrane and confers the toxicity to endotoxins. Its structure is highly conserved throughout all Gram-negative bacteria.

2. The hydrophilic inner core of the polysaccharide part of LPS, the **R-antigen**, is a short sugar chain with a highly conserved sequence. It is harboring a lot of negative charges and is thought to function as the main barrier against hydrophobic substances like antibiotics and detergents.

3. The hydrophilic and extremely variable outer polysaccharide, the **O-antigen**, is involved, for example in cell adherence and interactions with the immune system of the host, i.e., it is responsible for the immunological properties and virulence of the bacteria.

2.4.2 Quantification of endotoxins

Endotoxins can be measured in highly sensitive photometric tests (“Pyrochrome”, Associates of Cape Cod, Inc.) and are expressed in endotoxin units (EU). For plasmid preparations the endotoxin level is given in EU per µg plasmid. A concentration of 0.1 EU/µg is usually considered endotoxin-free.

2.4.3 Removal of endotoxins

Endotoxins are released from cells in small amounts during cell growth and in very large quantities upon cell death and lysis and thus also during plasmid purification. Like intact cells the free LPS molecules induce inflammatory reactions of the mammalian immune system. Therefore they have to be removed quantitatively from plasmid preparations to guarantee high transfection rates and high viability of transfected cells.

Due to their amphiphilic nature and their negative charge endotoxins behave like DNA and are co-purified with most common plasmid purification systems. Regular silica-membrane kits with a purification procedure based on chaotropic salt lead to plasmid DNA with an endotoxin level of > 1000 EU/µg. Anion exchange kits like **NucleoBond® Xtra** reduce endotoxins to a level of < 1 EU/µg. However, since this may be still too high for successful transfection of very sensitive cells like primary or neuronal cells, **NucleoBond® 96 Xtra EF** was developed to reduce the endotoxin level to < 0.05 EU/µg plasmid DNA using a patented procedure.

2.5 Growth of bacterial cultures

2.5.1 Culture media and volume

Yield and quality of plasmid DNA are highly dependent upon the **type of culture media** and antibiotics, the bacterial host strain, the plasmid type, size, and copy number, and also on **growth conditions**.

For standard high-copy plasmids, LB (Luria-Bertani) broth is recommended. However, due to the limited culture volume of 1–5 mL, rich media such as 2x YT (Yeast / Tryptone), TB (Terrific Broth), or CircleGrow can be used. In such rich media, bacteria grow faster, reach the stationary phase much earlier, and greater cell masses can be achieved compared to LB medium.

Cell cultures can either be grown directly in a Culture Plate, which is provided with the kit, or they can be cultivated in glass tubes.

The cell culture volume in the Culture Plate is limited to 1.2–1.5 mL of selective LB or rich medium in each well. Exceeding the 1.5 mL limit can lead to cross-contamination due to spillage during incubation. A single bacterial colony should be seeded in each well and the Culture Plate covered with the Gas-permeable Foil. The cultures can then be grown in a suitable incubator at 37 °C with vigorous shaking (200–400 rpm). The Culture Plate can be fixed to the shaker with large-size flask clamps (for 2 L flasks) or tape.

Cell growth is very slow under standard conditions due to bad oxygen supply and will take much longer to reach reasonable optical densities compared to oxygen saturated cultures. Additionally, even when the culture growth has slowed and seems to go stationary, plasmid production is still in progress, especially with high-copy constructs. Therefore, prolonging incubation time from the typical 14–20 h to 30–35 h may achieve higher plasmid yields (see Figure 3).

To reach even higher yields, well aerated glass tubes can be used instead of the 96-well culture plate. In that case, cultures are grown in up to 5 mL selective LB (16-24 h) or rich medium (10–14 h) at 37 °C in an appropriate shaker (200–250 rpm).

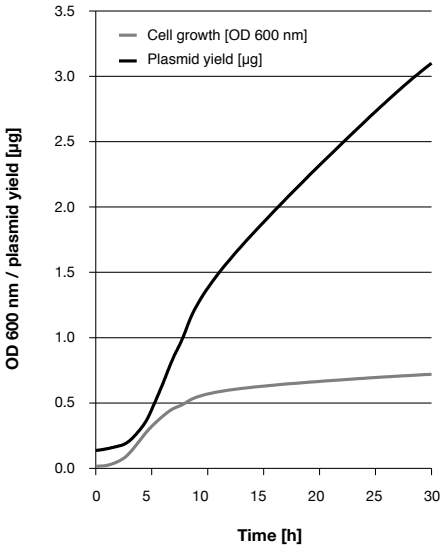


Figure 3: A 150 mL uniformly inoculated LB / *E. coli* DH5α/pcDNA3.1 culture was split into a 96-well culture plate (1.5 mL/well). The cultures were shaken at 250 rpm and 37 °C for up to 30 h. Cultures were harvested in triplicate every hour and subjected to plasmid purification.

Cells can be harvested by centrifugation for 10 min at 1,000 x *g*. In order to avoid cell pellets that are too tight to be easily dissolved, higher g-forces are not recommended. For cultures grown in glass tubes, cells can be harvested in the Culture Plate. Therefore, transfer only 1.5 mL of each culture to the Culture Plate, centrifuge, and discard the supernatant. Repeat these steps to pellet the whole 5 mL culture.

Residual medium can be removed by tapping tubes or plate upside down on a clean paper sheet or soft tissue.

Bacterial cultures should be grown under **antibiotic selection** at all times to ensure plasmid propagation. Without this selective pressure, cells tend to lose a plasmid during cell division. Since bacteria grow much faster without the burden of a high-copy plasmid, they take over the culture rapidly and the plasmid yield goes down regardless of the cell mass. Table 1 gives information on concentrations of commonly used antibiotics.

Table 1: Information about antibiotics according to Maniatis*			
Antibiotic	**Stock solution (concentration)**	**Storage**	**Working concentration**
Ampicillin	50 mg/mL in H_2O	-20 °C	20–50 µg/mL
Carbenicillin	50 mg/mL in H_2O	-20 °C	20–60 µg/mL
Chloramphenicol	34 mg/mL in EtOH	-20 °C	25–170 µg/mL
Kanamycin	10 mg/mL in H_2O	-20 °C	10–50 µg/mL
Streptomycin	10 mg/mL in H_2O	-20 °C	10–50 µg/mL
Tetracycline	5 mg/mL in EtOH	-20 °C	10–50 µg/mL

2.5.2 Host strain and plasmid copy-number

The quality of the plasmid DNA is mostly influenced by the ***E. coli* host strain** used. Whereas strains like DH5α™ or XL1-Blue usually produce high quality super-coiled plasmid DNA, other strains with high levels of endonuclease activity, such as HB101, might yield lower quality plasmid giving poor results in downstream applications like enzymatic restriction or sequencing.

The **type of plasmid**, especially its **size and the origin of replication (ori),** also has a crucial influence on DNA yield. In general, the larger the plasmid or the cloned insert, the lower the expected DNA yield is due to a lower copy number. Even a high-copy construct based on a ColE1 ori can behave like a low-copy vector if it contains a large or unfavorable insert. In addition, the ori itself influences the yield by a factor 10–100. For example, plasmids based on pBR322 or pACYC, cosmids or BACs are maintained at copy numbers less than 20, and can be as low as 1 copy per cell, whereas vectors based on pUC, pBluescript, or pGEM® can be present in several hundred copies per cell.

Therefore, all the above mentioned factors should be taken into consideration if a particular DNA yield is required. Culture volume and lysis procedures should be adjusted accordingly.

* Maniatis T, Fritsch EF, Sambrook J: *Molecular cloning. A laboratory manual*, Cold Spring Harbor, Cold Spring, New York 1982.

2.5.3 Chloramphenicol amplification of low-copy plasmids

To dramatically increase the low copy number of pMB1/colE1 derived plasmids grow the cell culture to mid or late log phase ($OD_{600} \approx 0.6–2.0$) under selective conditions with an appropriate antibiotic. Then add 170 µg/mL chloramphenicol and continue incubation for a further 8–12 hours. Chloramphenicol inhibits host protein synthesis and thus prevents replication of the host chromosome. Plasmid replication, however, is independent of newly synthesized proteins and continues for several hours until up to 2000–3000 copies per cell are accumulated*.

Alternatively, bacterial cultures can be grown with only partial inhibition of protein synthesis under lower chloramphenicol concentrations (10–20 µg/mL) resulting in a 5 to 10-fold greater yield of plasmid DNA**.

Both methods show the positive side effect of much less genomic DNA per plasmid, but they obviously work only with plasmids that do not carry the chloramphenicol resistance gene. Furthermore, the method is only effective with low copy number plasmids under stringent control (e.g., pBR322). All modern high copy number plasmids (e.g., pUC) are already under relaxed control due to mutations in the plasmid copy number control genes and show no significant additional increase in their copy number.

2.6 Lysate neutralization and LyseControl

Proper mixing of the lysate with Neutralization Buffer NEU-EF is of utmost importance for complete precipitation of SDS, protein, and genomic DNA. Incomplete neutralization leads to reduced yields, slower flow-rates, and potential clogging of the NucleoBond® Filter Plate. However, released plasmid DNA is very vulnerable at this point and shaking too much or too strongly will damage the DNA.

Therefore, **do not vortex or shake** but **invert the mixture very gently** until a fluffy off-white precipitate has formed and the **LyseControl** has turned **colorless** throughout the lysate without any traces of blue color.

* Maniatis T, Fritsch EF, Sambrook J: *Molecular cloning. A laboratory manual*, Cold Spring Harbor, Cold Spring, New York 1982.

** Frenkel L, Bremer H: *Increased amplification of plasmids pBR322 and pBR327 by low concentrations of chloramphenicol*, DNA (5), 539–544, 1986.

3 Storage conditions and preparation of working solutions

Attention:

All kit components can be stored at room temperature (18–25 °C) and are stable for at least one year. Storage of buffer LYS-EF below 20 °C may cause precipitation of SDS. If salt precipitate is observed, incubate buffer at 30–40 °C for several minutes and mix well until all precipitate is redissolved completely. Cool down to room temperature before use.

Before the first use of the **NucleoBond® 96 Xtra EF** kit, prepare the following:

- Dissolve the lyophilized RNase A by adding 1 mL of Buffer RES-EF. Wearing gloves is recommended. Pipette up and down until the RNase A is dissolved completely. Transfer the RNase A solution back to the bottle containing Buffer RES-EF and shake well. Note on the bottle the date of RNase A addition. The final concentration of RNase A is 60 µg/mL. Store Buffer RES-EF with RNase A at 4 °C. The solution will be stable at this temperature for at least 6 months.
- Add indicated volume of 96–100 % ethanol to the endotoxin-free water in the bottles labeled “80 % EtOH”

	NucleoBond® 96 Xtra EF	
	1 x 96 preps	**4 x 96 preps**
REF	**740430.1**	**740430.4**
80 % EtOH (Concentrate)	50 mL Add 200 mL ethanol	200 mL Add 800 mL ethanol to each bottle

4 Safety instructions

The following components of the **NucleoBond® 96 Xtra EF** kit contain hazardous contents.

Wear gloves and goggles and follow the safety instructions given in this section.

GHS classification

Only harmful features need not be labeled with H and P phrases until 125 mL or 125 g.

Mindergefährliche Eigenschaften müssen bis 125 mL oder 125 g nicht mit H- und P-Sätzen gekennzeichnet werden.

Component *Inhalt*	Hazard contents *Gefahrstoff*	GHS symbol *GHS Symbol*	Hazard phrases *H-Sätze*	Precaution phrases *P-Sätze*
LYS-EF	Sodium hydroxide < 2 % *Natriumhydroxid < 2 %*	Warning *Achtung*	290, 315, 319	234, 280, 302+352, 305+351+338, 332+313, 337+313, 390, 406
EQU-EF, WASH-EF	Buffer salts + ethanol 5–20% *Puffersalze + Ethanol 5–20%*	Warning *Achtung*	226	210, 233, 403+235
ELU-EF	Buffer salts + isopropanol 10–15% *Puffersalze + Isopropanol 10–15%*	Warning *Achtung*	226, 319	210, 233, 280, 305+351+338, 337+313, 403+235
RNase A	RNase A, lyophilized *RNase A, lyophilisiert*	Danger *Gefahr*	317, 334	261, 280, 302+352, 304+341, 333+313, 342+311, 363

Hazard phrases

H 226	Flammable liquid and vapour. *Flüssigkeit und Dampf entzündbar.*
H 290	May be corrosive to metals. *Kann gegenüber Metallen korrosiv sein.*
H 315	Causes skin irritation. *Verursacht Hautreizungen.*
H 317	May cause an allergic skin reaction. *Kann allergische Hautreaktionen verursachen.*
H 319	Causes serious eye irritation. *Verursacht schwere Augenreizung.*
H 334	May cause allergy or asthma symptoms or breathing difficulties if inhaled. *Kann bei Einatmen Allergie, asthmaartige Symptome oder Atembeschwerden verursachen.*

Precaution phrases

P 210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking. *Von Hitze, heißen Oberflächen, Funken, offenen Flammen sowie anderen Zündquellenarten fernhalten. Nicht rauchen.*
P 233	Keep container tightly closed. *Behälter dicht verschlossen halten.*
P 234	Keep only in original container. *Nur im Originalbehälter aufbewahren.*
P 261	Avoid breathing dust. *Einatmen von Staub vermeiden.*
P 280	Wear protective gloves / eye protection. *Schutzhandschuhe / Augenschutz tragen.*
P 302+352	IF ON SKIN: Wash with plenty of water/… *BEI KONTAKT MIT DER HAUT: Mit viel Wasser/… waschen.*
P 304+341	IF INHALED: If breathing is difficult, remove to fresh air and keep at rest in a position comfortable for breathing. *Bei Einatmen: Bei Atembeschwerden an die frische Luft bringen und in einer Position ruhigstellen, die das Atmen erleichtert.*
P 305+351+338	IF IN EYES: Rinse continuously with water for several minutes. Remove contact lenses if present and easy to do – continue rinsing. *BEI KONTAKT MIT DEN AUGEN: Einige Minuten lang behutsam mit Wasser spülen. Vorhandene Kontaktlinsen nach Möglichkeit entfernen. Weiter spülen.*
P 332+313	IF skin irritation occurs: Get medical advice / attention. *Bei Hautreizung: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 333+313	If skin irritation occurs: Get medical advice / attention. *Bei Hautreizung: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 337+313	Get medical advice / attention *Bei anhaltender Augenreizung: Ärztlichen Rat einholen / ärztliche Hilfe hinzuziehen.*
P 342+311	If experiencing respiratory symptoms: Call a POISON CENTER / doctor /… *Bei Symptomen der Atemwege: GIFTINFORMATIONSZENTRUM / Arzt /… anrufen.*
P 363	Wash contaminated clothing before reuse *Kontaminierte Kleidung vor erneutem Tragen waschen.*
P 390	Absorb spillage to prevent material damage. *Verschüttete Mengen aufnehmen, um Materialschäden zu vermeiden.*
P 403+235	Store in a well ventilated place. Keep cool. *Kühl an einem gut belüfteten Ort aufbewahren.*
P 406	Store in a corrosive resistant /… container with a resistant inner liner. *In korrosionsbeständigem / (...) Behälter mit korrosionsbeständiger AUskleidung aufbewahren.*

For further information please see Material Safety Data Sheets ***(www.mn-net.com).***
Weiterführende Informationen finden Sie in den Sicherheitsdatenblättern ***(www.mn-net.com)****.*

5 Protocols

5.1 NucleoBond® 96 Xtra EF – manual vacuum processing

Before starting the preparation:

- Check that Buffer RES-EF and 80 % ethanol were prepared according to section 3.

Protocol-at-a-glance

1	**Cultivate** and **harvest** bacterial cells	**1.5–5 mL cell culture** **1,000 x *g*, 10 min**
2	**Resuspend** bacterial cells	**400 µL RES-EF** **Mix or shake**
3	**Lyse** bacterial cells	**400 µL LYS-EF** **RT, 2–5 min**
4	**Neutralize**	**400 µL NEU-EF** **Mix or shake**
5	**Assemble** vacuum manifold filtration set-up (see page 21)	
6	**Transfer** crude lysates onto NucleoBond® Filter Plate (light orange rings)	
7	**Clear** crude lysates by vacuum filtration	**Apply vacuum (-0.4 to -0.6 bar*), 1–5 min**
8	**Assemble** vacuum manifold Xtra purification set-up (see page 21)	
9	**Equilibrate** NucleoBond® Xtra EF Plate	**900 µL EQU-EF** **Gravity flow**
10	**Load** cleared lysates onto NucleoBond® Xtra EF Plate	**Gravity flow**

* Reduction of atmospheric pressure

11	**Wash** NucleoBond® Xtra EF Plate	
	1st wash	**900 µL ENDO-EF** **Gravity flow**
	2nd wash	**900 µL ENDO-EF** **Gravity flow**
	3rd wash	**900 µL WASH-EF** **Gravity flow**
12	**Assemble** vacuum manifold Xtra elution set-up (see page 21)	
13	**Elute** DNA from NucleoBond® Xtra EF Plate	**500 µL ELU-EF** **Gravity flow**
14	**Assemble** vacuum manifold Finalizer purification set-up (see page 21)	
15	**Precipitate** plasmid DNA	**350 µL isopropanol (room temperature)** **RT, 5 min**
16	**Equilibrate** NucleoBond® Finalizer Plate (red rings)	**1 mL TE-EF** **Apply vacuum (-0.2 to -0.4 bar*)**
17	**Load** precipitated plasmid DNA onto NucleoBond® Finalizer Plate	**Apply vacuum (-0.2 to -0.4 bar*)**
18	**Wash** NucleoBond® Finalizer Plate	
	1st wash	**1 mL 80 % EtOH** **Apply vacuum (-0.2 to -0.4 bar*)**
	2nd wash	**1 mL 80 % EtOH** **Apply vacuum (-0.2 to -0.4 bar*)**
19	**Assemble** vacuum manifold Finalizer drying set-up (see page 21)	

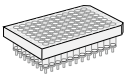
* Reduction of atmospheric pressure

20	**Dry** NucleoBond® Finalizer Plate	**Apply vacuum (-0.4 to -0.6 bar*), 5–10 min**
21	**Assemble** vacuum manifold Finalizer elution set-up (see page 21)	
22	**Elute** plasmid DNA from NucleoBond® Finalizer Plate	**100–200 µL TE-EF or H_2O-EF** **RT, 1–3 min** **Apply vacuum (max. 0.4 bar*), 1 min**

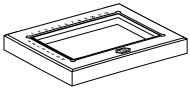
* Reduction of atmospheric pressure

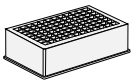
Setup of vacuum manifold:

Filtration setup

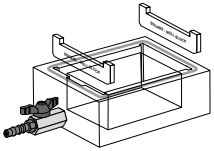


NucleoBond® Filter Plate (light orange rings)





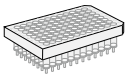
Square-well Block



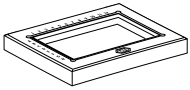
Manifold base with spacers ‘SQUARE-WELL BLOCK’ inserted

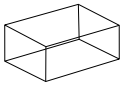
Xtra purification setup

Xtra elution setup

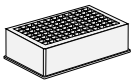


NucleoBond® Xtra EF Plate

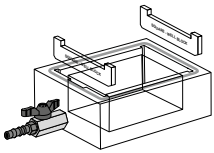




Waste Container



Square-well Block

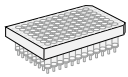


Manifold base with spacers ‘SQUARE-WELL BLOCK’ inserted

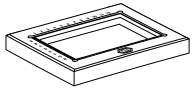
Finalizer purification setup

Finalizer drying setup

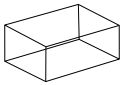
Finalizer elution setup



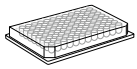
NucleoBond® Finalizer Plate (red rings)



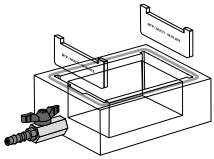
MN Wash Plate



Waste Container



Elution Plate U-bottom



Manifold base with spacers ‘MTP/MULTI-96 PLATE’ inserted

Detailed protocol

Processing NucleoBond® 96 Xtra EF under vacuum requires the NucleoVac 96 Vacuum Manifold.

Before starting the preparation:

- Check that Buffer RES-EF and 80 % EtOH were prepared according to section 3.

1 Cultivate and harvest bacterial cells

Grow and harvest the bacterial cells as described in section 2.5.

2 Resuspend bacterial cells

Add **400 µL Buffer RES-EF with RNase A** to each sample. Resuspend the cells completely by pipetting up and down or vortexing.

For an efficient lysis it is important that no clumps remain in suspension.

3 Lyse bacterial cells

Check Lysis Buffer LYS-EF for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30–40 °C until precipitate is completely dissolved. Cool buffer to room temperature (18–25 °C) before use.

Add **400 µL Buffer LYS-EF** to the suspension.

Incubate at **room temperature** for a maximum of **5 min** with moderate shaking (300 rpm).

Warning: Do not vortex, as this will shear and release contaminating chromosomal DNA from cellular debris into the suspension. Do not allow the lysis reaction to proceed for more than 5 minutes, as prolonged exposure to alkaline conditions can irreversibly denature and degrade plasmid DNA and liberate contaminating chromosomal DNA.

4 Neutralize

Add **400 µL Buffer NEU-EF** to the suspension. For lysis in tubes, close the tubes and mix by inverting several times. For lysis in plates, mix by pipetting up and down very slowly and carefully after addition of Buffer NEU-EF.

Mix the lysate until the blue color completely disappears to precipitate protein and chromosomal DNA, and to completely renature plasmid DNA.

Optional: Incubate on ice for 5 min for optimal formation of precipitate.

5 Assemble vacuum manifold filtration set-up

Insert spacers labeled ‘Square-well Block’ notched side up into the grooves located on the short sides of the **NucleoVac 96 Vacuum Manifold**. Place a new Square-well Block inside the manifold. Close the manifold with the manifold lid. Place the **NucleoBond® Filter Plate** on top of the manifold lid (see page 21).

6 Transfer crude lysates onto NucleoBond® Filter Plate

Mix the crude lysates from step 4 by pipetting the entire volume up and down once. Transfer the lysates completely onto the NucleoBond® Filter Plate.

7 Clear crude lysate by vacuum filtration

Apply vacuum of **-0.4 to -0.6 bar* (1–5 min)**. If necessary, press down the NucleoBond® Filter Plate slightly until the flow starts. Adjust vacuum to establish a flow rate of 1–2 drops per second.

Release the vacuum when the crude lysate has passed the NucleoBond® Filter Plate.

8 Assemble vacuum manifold Xtra purification set-up

Discard the NucleoBond® Filter Plate. Take off the manifold lid. Remove the Square-well Block with cleared lysates. Insert the waste container into the manifold base. Close the manifold with the manifold lid. Place the **NucleoBond® Xtra EF Plate** on top of the manifold lid (see page 21).

9 Equilibrate NucleoBond® Xtra EF Plate

***Attention*: step 9–13 are performed without applying any vacuum!**

Load **900 µL Buffer EQU-EF** to each well and allow the buffer to pass the resin by gravity flow.

10 Load cleared lysates onto NucleoBond® Xtra EF Plate

After Buffer EQU-EF has completely run through, load the **cleared lysates** from step 7 and allow the samples to pass the resin by gravity flow.

11 Wash NucleoBond® Xtra EF Plate

1st wash

After the cleared lysates have run through, add **900 µL Buffer ENDO-EF** to each well. Allow the buffer to pass the resin by gravity flow.

* Reduction of atmospheric pressure

2nd wash

Repeat washing step with **900 µL Buffer ENDO-EF**.

3rd wash

Repeat washing step with **900 µL Buffer WASH-EF**.

12 Assemble vacuum manifold Xtra elution set-up

Take off the manifold lid and the NucleoBond® Xtra EF Plate. Remove waste container and insert a new Square-well Block. Place the manifold lid with the inserted NucleoBond® Xtra EF Plate back onto the manifold base (see page 21).

13 Elute DNA from NucleoBond® Xtra EF Plate

Load **0.5 mL Buffer ELU-EF** to each well and collect the eluted plasmid DNA by gravity flow.

14 Assemble vacuum manifold Finalizer purification set-up

Insert spacers 'MTP/Mult-96 Plate', notched side up, into the grooves located on the short sides of the manifold. Insert waste container into the manifold base. Insert the MN Wash Plate on the spacers inside the manifold base. Close the manifold base with the manifold lid. Place the NucleoBond® Finalizer Plate on top of the manifold lid (see page 21).

15 Precipitate plasmid DNA

Add **350 µL room temperature isopropanol** to each well of the Square-well Block with the eluted DNA to precipitate the plasmid. Mix by repeated pipetting up and down. Incubate for 5 min at room temperature.

16 Equilibrate NucleoBond® Finalizer Plate

***Attention*: Be sure to use the shortest vacuum-time possible to avoid filtration of airborne bacteria onto the silica membrane.**

Add **1 mL Buffer TE-EF** to each well and apply vacuum **(-0.2 to -0.4 bar*)** until all liquid has passed the silica membrane.

* Reduction of atmospheric pressure

17 Load precipitated plasmid DNA onto NucleoBond® Finalizer Plate

Load the mixture from step 15 to the NucleoBond® Finalizer Plate and apply vacuum **(-0.2 to -0.4 bar*)** until all liquid has passed the silica membrane.

18 Wash NucleoBond® Finalizer Plate

1st wash

Add **1 mL 80 % EtOH** to each well and apply vacuum **(-0.2 to -0.4 bar*)** until all liquid has passed the silica membrane.

2nd wash

Repeat washing step with **1 mL 80 % EtOH**.

19 Assemble vacuum manifold Finalizer drying step

Take off the manifold lid and the NucleoBond® Finalizer Plate. Dry the outlets of the NucleoBond® Finalizer Plate by placing it on a sheet or filter paper or soft tissue. Remove the MN Wash Plate and the waste container.

Close the manifold with the manifold lid and place the NucleoBond® Finalizer Plate back onto the manifold lid (see page 21).

20 Dry NucleoBond® Finalizer Plate

Apply vacuum **(-0.4 to -0.6 bar*)** for **5–10 min** to dry the membrane completely. Run vacuum pump continuously. Typically the adjusted vacuum is not reached at this step. It is more important to have a continuous air-flow to evaporate the ethanol.

Note: The ethanol inhibits enzymatic reactions and has to be removed completely before eluting DNA.

Finally, release the vacuum.

21 Assemble vacuum manifold Finalizer elution set-up

Take off the manifold lid and the NucleoBond® Finalizer Plate. Insert the Elution Plate U-bottom on the spacers inside the manifold base. Close the manifold with the manifold lid. Place the NucleoBond® Finalizer Plate back onto of the manifold lid (see page 21).

* Reduction of atmospheric pressure

22 Elute plasmid DNA from NucleoBond® Finalizer Plate

Add **100–200 µL Buffer TE-EF** or **H_2O-EF** to each well. The elution buffer should be dispensed carefully onto the center of the silica membrane. Incubate the buffer on the membrane for **1–3 min** at **room temperature**.

Apply vacuum of **max. -0.4 bar* (1 min)**. If necessary, press down the NucleoBond® Finalizer Plate slightly and collect the eluted DNA. After the elution buffer has passed the wells, release vacuum.

Note: To increase final yield, reapply the first eluate to the NucleoBond® Finalizer Plate and elute a second time. Alternatively, use 100–200 µL of fresh Buffer TE-EF / H_2O-EF for a second elution. Furthermore, heating elution buffer to 70 °C can improve elution especially for DNA > 5–10 kbp.

Remove the Elution Plate U-bottom containing the eluted DNA and seal the plate with adhesive cover foil for further storage.

* Reduction of atmospheric pressure

5.2 NucleoBond® 96 Xtra EF – manual centrifuge processing

To run the NucleoBond® Filter Plate (clearing the lysate) and NucleoBond® Finalizer Plate (final clean-up), the vacuum procedure can be replaced by centrifugation steps.

Required hardware and consumables:

- A microtiter plate centrifuge which is able to accommodate the NucleoBond® Filter / Finalizer Plates stacked on a Square-well Block or a Rack of Tube Strips (bucket height 85 mm) .
- Rack of Tube Strips for elution (see ordering information)
- Additional MN Square-well Block for collection of flow-through (see ordering information)

Before starting the preparation:

- Check that Buffer RES-EF and 80 % ethanol were prepared according to section 3.

---

1 Cultivate, harvest and lyse bacterial cells

Follow steps 1–4 of the main protocol 5.1 for manual vacuum processing.

---

2 Clear crude lysate

Place the NucleoBond® Filter Plate on top of a MN Square-well Block.

Mix the crude lysates by pipetting the entire volume up and down once.

Transfer the lysates completely onto the NucleoBond® Filter Plate and centrifuge for **5 min** at **maximum speed**.

---

3 Purify plasmid with NucleoBond® Xtra EF Plate and precipitate DNA

Follow steps 8–14 of the main protocol 5.1 for manual vacuum processing.

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4 Equilibrate NucleoBond® Finalizer Plate

Place the NucleoBond® Finalizer Plate on top of a MN Square-well Block (not included). Add **1 mL Buffer TE-EF** to each well and centrifuge for **2 min** at **maximum speed**.

Discard the flow-through and place the NucleoBond® Finalizer Plate back on top of the MN Square-well Block.

---

5 Load precipitated plasmid DNA onto NucleoBond® Finalizer Plate

Load the mixture from step 3 to the NucleoBond® Finalizer Plate and centrifuge for **2 min** at **maximum speed**.

Discard the flow-through and place the NucleoBond® Finalizer Plate back on top of the MN Square-well Block.

6 Wash NucleoBond® Finalizer Plate

1st wash

Add **1 mL 80 % EtOH** to each well and centrifuge for **2 min** at **maximum speed**.

Discard the flow-through and place the NucleoBond® Finalizer Plate back on top of the MN Square-well Block.

2nd wash

Repeat washing step with **1 mL 80 % EtOH**.

7 Dry NucleoBond® Finalizer Plate

Centrifuge for **10 min** at **maximum speed**.

8 Elute plasmid DNA from NucleoBond® Finalizer Plate

Place the NucleoBond® Finalizer Plate on top of a Rack of Tube Strips (not included). Add **100–200 µL Buffer TE-EF** or **H_2O-EF** to each well. The elution buffer should be dispensed carefully onto the center of the silica membrane. Incubate for 1–3 min at room temperature.

Centrifuge for **2 min** at **maximum speed**.

To increase final yield, reapply the first eluate to the NucleoBond® Finalizer Plate and elute a second time. Alternatively, **use 100–200 µL of fresh Buffer TE-EF / H_2O-EF** for a second elution. Furthermore, heating the elution buffer to 70 °C can improve elution especially for DNA > 5–10 kbp.

Note: Do not use a microtiter plate as elution plate. Microtiter plates may crack under centrifugation at > 1,500 x g.

6 Appendix

6.1 Troubleshooting

Problem	Possible cause and suggestions
No or low plasmid DNA yield	*Plasmid did not propagate* • Bacteria tend to lose their plasmids if not enough selective pressure by appropriate antibiotics is applied. Use only fresh colonies and cultures for inoculation. Always use enough fresh antibiotics in culture plates and media. *Incomplete cell lysis* • Cells are not completely resuspended. Use only 1000 x *g* to pellet cells in order to obtain soft pellets. Increase shaking, shaking time, or resuspend by pipetting up and down. • SDS precipitates in Lysis Buffer LYS-EF when stored below 20 °C. Heat buffer to 30–40 °C for several minutes until all precipitate is dissolved. Cool down Buffer LYS-EF to room temperature before use. • Too much cell mass was used for the given lysis buffer volumes. Increase lysis buffer volumes or reduce culture volume for optimal lysis. • Resuspended cells are not mixed efficiently with lysis buffer. Increase shaking during lysis incubation but do not vortex or pipette to avoid shearing of genomic DNA. Alternatively, seal the plate with self-adhesive foil and invert the plate 5–6 times. Be careful to avoid cross-contamination when removing the foil. *Incomplete neutralization after cell lysis* • Increase shaking, shaking time, or mix by pipetting up and down very, very gently. Use wide bore pipette tips to avoid shearing of genomic DNA. Alternatively, seal the plate with self-adhesive foil and invert the plate 10–15 times. Be careful to avoid cross-contamination when removing the foil. *Wrong elution buffers used* • Binding to the NucleoBond® Xtra EF Plate is based on anion-exchange chemistry. Elution has to be done with the provided high-salt ELU-EF elution buffer. TE-EF or H_2O-EF are for elution of the NucleoBond® Finalizer Plate only.

Problem	Possible cause and suggestions
No or low plasmid DNA yield *(continued)*	*NucleoBond® Xtra EF Plate was used under vacuum* • Incomplete binding and elution of DNA to anion-exchange resin. NucleoBond® Xtra EF Plate must be run with gravity flow only. *No ethanol was added to 80 % EtOH wash buffer* • DNA was eluted prematurely from the NucleoBond® Finalizer Plate during the washing step. Add appropriate amount of 96–100 % ethanol to wash buffer concentrate. *Inefficient elution from NucleoBond® Finalizer Plate* • When using other buffers for elution than the provided TE-EF or H_2O-EF make sure pH is higher than 7. • The NucleoBond® Finalizer Plate can only be used for plasmids, ideally < 15 kbp. Larger constructs such as cosmids or BACs show much lower elution efficiency and tend to be damaged during the procedure. *Low-copy plasmid was used* • Increase cell culture volume and lysis buffer volume.
NucleoBond® Xtra EF or Filter Plate is clogged	*See "No or low plasmid DNA yield" – "Incomplete neutralization after cell lysis"* *Cleared lysates were stored before loading onto the NucleoBond® Xtra EF Plate* • More fine precipitate can form upon storage, especially at lower temperatures. Do not store cleared lysates but proceed immediately with the loading step.
Genomic DNA contamination	*Excessive mixing after addition of Buffer LYS-EF or NEU-EF* • Vortexing or pipetting can shear genomic DNA which is copurified with plasmid DNA. Reduce number of mixing cycles, reduce shaker speed and shaking time. Use only wide bore pipette tips for pipetting gently up and down. *Genomic DNA was sheared during crude lysate transfer to the NucleoBond® Filter Plate* • Use wide bore pipette tips only. Pipette very slowly.

Problem	Possible cause and suggestions
Genomic DNA contamination *(continued)*	*Lysis time too long* • Genomic DNA and plasmid DNA start to degrade and will be copurified. Lysis time must not exceed 5 min. *Cell culture grown to late stationary or starvation phase* • Large amounts of dead cells are a source of genomic DNA fragments. Reduce culture incubation time, especially when using rich culture media.
RNA contamination	*No or low RNase A activity* • RNase A was not added to Buffer RES-EF. Dissolve RNase A in Buffer RES-EF and store buffer at 4 °C. *Insufficient washing* • If plasmid yield is low, much unused binding capacity leads to tighter binding of RNA. Double or triple washing steps.
Plasmid DNA does not perform well in downstream application	*Carry-over of ethanol* • Silica membrane of the NucleoBond Finalizer Plate was not dry before elution or residual wash buffer droplets inside or outside the outlets. Tap the NucleoBond Finalizer Plate onto a filter paper or soft tissue to soak up residual liquid. Then dry NucleoBond Finalizer Plate under vacuum for at least 5–10 min. *EDTA in Elution Buffer TE-EF* • EDTA may inhibit enzymatic reactions like DNA sequencing. Use H_2O-EF for elution. *DNA is irreversibly denatured* • Denatured plasmid DNA typically runs faster on agarose gels than supercoiled DNA. Do not lyse the sample after addition of Buffer LYS-EF for more than 5 min.

Problem	Possible cause and suggestions
Endotoxin level too high	*Too much cell mass was used* • Increase lysis buffer volume or reduce culture volume. *Inefficient endotoxin removal* • Make sure to wash two times with Buffer ENDO-EF. *Contamination of DNA after purification* • Use only new pyrogen- or endotoxin-free consumables and plastics. Endotoxins tend to stick to glass and are hard to remove. If glass-ware is used, heat over night at 180 °C to destroy endotoxins. Autoclaving does not inactivate endotoxins and is not recommended if the autoclave is also used for inactivation of bacterial waste. • Use only the endotoxin-free buffers provided with the kit, especially for preparation of 70 % ethanol and TE-EF or H_2O-EF for elution.

6.2 Ordering information

Product	REF	Pack of
NucleoBond® 96 Xtra EF	740430 .1 740430 .4 740430 .24	1 x 96 preps 4 x 96 preps 24 x 96 preps
Buffer RES-EF (without RNase A)	740386 .1000	1 L
Buffer LYS-EF	740387 .1000	1 L
Buffer NEU-EF	740388 .1000	1 L
Buffer ENDO-EF	740391 .1000	1 L
Buffer WASH-EF	740392 .1000	1 L
Buffer ELU-EF	740393 .1000	1 L
RNase A (lyophilized)	740505	100 mg

Product	REF	Pack of
NucleoVac 96 Vacuum Manifold	740681	1
NucleoVac Vacuum Regulator	740641	1
Round-well Block with Cap Strips	740475 740475 .24	4 sets 24 sets
Rack of Tube Strips (1 set consists of 1 rack, 12 strips with 8 tubes each, and 12 Cap Strips)	740477 740477 .24	4 sets 24 sets
Cap Strips	740478 740478 .24	48 288
MN Square-well Block	740476 740476 .24	4 24
MN Wash Plate	740479 740479 .24	4 24
Culture Plate (with Gas-permeable Foil)	740488 740488 .24	4 sets 24 sets
Elution Plate U-bottom (with Self-adhering Foil)	740486 .24	24 sets
Gas-permeable Foil	740675	50
Self-adhering Foil	740676	50

Visit ***www.mn-net.com*** for more detailed product information.

6.3 Product use restriction/warranty

NucleoBond® 96 Xtra EF components are intended, developed, designed, and sold FOR RESEARCH PURPOSES ONLY, except, however, any other function of the product being expressly described in original MACHEREY-NAGEL product leaflets.

MACHEREY-NAGEL products are intended for GENERAL LABORATORY USE ONLY! MACHEREY-NAGEL products are suited for QUALIFIED PERSONNEL ONLY! MACHEREY-NAGEL products shall in any event only be used wearing adequate PROTECTIVE CLOTHING. For detailed information please refer to the respective Material Safety Data Sheet of the product! MACHEREY-NAGEL products shall exclusively be used in an ADEQUATE TEST ENVIRONMENT. MACHEREY-NAGEL does not assume any responsibility for damages due to improper application of our products in other fields of application. Application on the human body is STRICTLY

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DNA/RNA/PROTEIN purification products of MACHEREY-NAGEL are suitable for *IN VITRO*-USES ONLY!

ONLY MACHEREY-NAGEL products specially labeled as IVD are also suitable for *IN VITRO*-diagnostic use. Please pay attention to the package of the product. *IN VITRO*-diagnostic products are expressly marked as IVD on the packaging.

IF THERE IS NO IVD SIGN, THE PRODUCT SHALL NOT BE SUITABLE FOR *IN VITRO*-DIAGNOSTIC USE!

ALL OTHER PRODUCTS NOT LABELED AS IVD ARE NOT SUITED FOR ANY CLINICAL USE (INCLUDING, BUT NOT LIMITED TO DIAGNOSTIC, THERAPEUTIC AND/OR PROGNOSTIC USE).

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MACHEREY-NAGEL shall only be responsible for the product specifications and the performance range of MN products according to the specifications of in-house quality control, product documentation and marketing material.

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